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Reverse Mutation Test using Microorganisms

Test number : 12923

Name of test substance :

June 28, 2005

QUALITY ASSURANCE STATEMENT

Title : Reverse mutation test of. microorganisms

Test number : 12923

Testing facility :

I hereby certify that this test is conducted according to Japanese Good Laboratory Practice, "Notification for concerning testing facilities conducting the test related to the new chemical substances (Notification No. 1121003 of JMHLW, No. 3 of JMETI, No. 031121004 of JMOE on November 21, 2003)," and to Japanese test guideline, "Notification of testing methods relating to the new chemical substances (Notification No. 1121002 of JMHLW, No. 2 of JMETI, No. 031121002 of JMOE on November 21, 2003)." and to OECD Guideline for Testing of Chemicals 471, "Bacterial Reverse Mutation Test (July 21, 1997)." The circumstances of inspections are as follows :

Inspection Date	Item of Inspection	Study director's Reported date	Manager's Reported date
April 26, 2005	Protocol	April 26, 2005	April 26, 2005
May 2, 2005	Observation	May 2, 2005	May 2, 2005
May 13, 2005	Operation	May 13, 2005	May 13, 2005
May 23, 2005	Observation	May 23, 2005	May 23, 2005
June 3, 2005	Final report (Draft)	June 3, 2005	June 3, 2005
June 28, 2005	Final report	June 28, 2005	June 28, 2005

Manager of Quality Assurance Unit

June 28, 2005

QUALITY ASSURANCE STATEMENT

Title : Reverse mutation test of microorganisms

Test number : 12923

Testing facility :

I hereby certify that this test is conducted according to Japanese Good Laboratory Practice, "The standard that the Minister of Labor establishes based on the Industrial Safety and Health Law Article 34-3 Paragraph 2 (Notification No. 76 of JMOL on September 1, 1988) and Notification on partial revision of the standard (Notification No. 13 of JMOL on March 29, 2000)," and to Japanese test guideline, "The standard that the Minister of Labor establishes based on the Industrial Safety and Health Law Article 57-2 Paragraph 1 (Notification No. 77 of JMOL on September 1, 1988) and Notification on partial revision of the standard (Notification No. 67 of JMOL on June 2, 1997)." and to OECD Guideline for Testing of Chemicals 471, "Bacterial Reverse Mutation Test (July 21, 1997)." The circumstances of inspections are as follows :

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May 23, 2005	Observation	May 23, 2005	May 23, 2005
June 3, 2005	Final report (Draft)	June 3, 2005	June 3, 2005
June 28, 2005	Final report	June 28, 2005	June 28, 2005

Manager of Quality Assurance Unit

June 28, 2005

Summary

The mutagenic activity of Ammonium was examined in the reverse mutation test by using bacterial strains that have two different patterns of the mutation. One of mutation is the base-pair substitution of *Salmonella typhimurium* TA100, TA1535 and *Escherichia coli* WP2 *uvrA*, and another mutation is the frameshift mutation of *Salmonella typhimurium* TA98 and TA1537. The reverse mutation test was composed of the preliminary, the main and the confirmatory test in accordance with the test guideline applied, and the reappearance of these test results was confirmed. These tests were performed in pre-incubation methods in all bacterial strains in both the presence and the absence of metabolic activation. In this test, two statistical analyses that are Dunnett's multiple comparison method (one-side test) and linear regression method were used to evaluate the test results. The number of the revertant colonies of each bacterial strain at each dose in the main and the confirmatory test was compared with the negative control in both the presence and the absence of metabolic activation, and statistically significant difference in the number of the revertant colonies between those two groups was analyzed first by the multiple comparison method. When the statistically significant difference was obtained by the multiple comparison method, the specific dose-relativity was analyzed by linear regression method. The test substance was judged positive for mutagenic activity when biologically significant increases such as significant increases in the number of the revertant colonies with clear dose-relativity and reappearance was obtained. The results of the test are as follows :

1. The test substance did not show statistically significant dose-related increase in the number of the revertant colonies compared with the negative control in any bacterial strains regardless of the presence or the absence of metabolic activation. In addition, the reappearance of the test results between the main and the confirmatory test was confirmed. Therefore, it was thought that the biologically significant increases were not observed in any bacterial strains and that the test substance was not inducible for mutagenicity.

2. The values of the negative controls and the positive controls were appropriate in comparison with the historical data of our laboratory. Furthermore, all of the positive controls, such as 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, sodium azide, 9-aminoacridine, benzo[*a*]pyrene and 2-aminoanthracene, increased the number of the revertant colonies two-fold or more compared with the negative control in all bacterial strains, respectively. These results indicate that the test has been properly carried out.

3. From the foregoing results, it is concluded that the mutagenic activity of is considered negative under the test conditions employed.

Title : Reverse mutation test of microorganisms.

Test number : 12923

Purpose : The objective of this test is to evaluate the mutagenic activity of the test substance using *Salmonella typhimurium* TA strains and *Escherichia coli* WP2 *uvrA*.

Sponsor :

Testing facility :

Duration of the test :

Test initiation date	April 26, 2005
Preliminary reverse mutation test initiation date	April 27, 2005
Reverse mutation test initiation date	May 12, 2005
Confirmatory reverse mutation test initiation date	May 19, 2005
Confirmatory reverse mutation test completion date	May 23, 2005
Final report draft preparation date	June 3, 2005
Test completion date	June 28, 2005

Personnel engaged in this test :

Facility Manager

Study Director

Study Personnel

Preparation of the final report, signature and statement :

I hereby state that this test was conducted in accordance with following Good Laboratory Practice and Test Guideline. The Good Laboratory Practice and Test guidelines that were applied in this tests are as follows.

< Good Laboratory Practice >

1. The standard that the Minister of Labor establishes based on the Industrial Safety and Health Law Article 34-3 Paragraph 2 (Notification No. 76 of JMOL on September 1, 1988) and Notification on partial revision of the standard (Notification No. 13 of JMOL on March 29, 2000).
2. Notification for concerning testing facilities conducting the test related to the new chemical substances (Notification No. 1121003 of JMHLW, No. 3 of JMETI, No. 031121004 of JMOE on November 21, 2003).

< Test guidelines >

1. The standard that the Minister of Labor establishes based on the Industrial Safety and Health Law Article 57-2 Paragraph 1 (Notification No. 77 of JMOL on September 1, 1988) and Notification on partial revision of the standard (Notification No. 67 of JMOL on June 2, 1997).
2. Notification of testing methods relating to the new chemical substances (Notification No. 1121002 of JMHLW, No. 2 of JMETI, No. 031121002 of JMOE on November 21, 2003).
3. OECD Guideline for Testing of Chemicals 471, "Bacterial Reverse Mutation Test" (21 July, 1997).

Study director
Division of Mutagenicity

June 28, 2005

1. Test materials

1.1 Test substance

4) Purity : 99.5%
5) Impurities : water, 0.5%
6)
7)
8) Appearance at ordinary temperature : white solid
9) Stability : stable in room temperature
10) Stability in solvent : stable in water, dimethylsulfoxide and acetone
11) Solubility : 10 % or more (water, dimethylsulfoxide and acetone)
12) Store conditions : in room temperature, shield from light
13) Lot number : RS4-56

1.2 Solvent

1) Name : distilled water
2) Supplier : Otsuka Pharmaceutical Factory, Inc.
3) Lot number : K4B78

1.3 Control substances

Distilled water was used as the negative control substance. The following known mutagens were used as the positive control substance :

AF-2	2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide (Wako Pure Chemical Industries, Ltd., Lot number SEL1402, Purity 99.0 wt%)
NaN_3	sodium azide (Wako Pure Chemical Industries, Ltd., Lot number ELE2329, Purity 99.2 wt%)
9AA	9-aminoacridine (SIGMA, Lot number 106F06681, Purity 97 wt%)
2AA	2-aminoanthracene (Wako Pure Chemical Industries, Ltd., Lot number ELJ6826, Purity 97.4 wt%)
B[*a*]P	benzo[*a*]pyrene (Wako Pure Chemical Industries, Ltd., Lot number ELQ2013, Purity 99.8 wt%)

1.4 Bacterial strains

1.4.1 Tester strains

The bacterial strains that have two different patterns of the mutation were used in this test. One is the base-pair substitution of *Salmonella typhimurium* TA100, TA1535 and *Escherichia coli* WP2 *uvrA*. Another is the frameshift mutation of *Salmonella typhimurium* TA98 and TA1537. *Salmonella typhimurium* TA strains were obtained on May 2, 1988 from Dr. Bruce N. Ames (University of California) and *Escherichia coli* WP2 *uvrA* was obtained on May 11, 1989 from Institute of Medical Science (University of Tokyo). All bacterial strains obtained were suspended in Nutrient broth No.2 (OXOID) and stored at -80℃.

Before the examination, the bacterial strains were reisolated from the frozen suspension by streaking it on a Nutrient broth agar plate and incubated at 37℃. The grown reisolated colonies were then resuspended in a Nutrient broth No.2 (OXOID) and incubated at 37℃ with shaking until these bacterial strains were attained to the early stationary phase. After incubation, appropriate volume of dehydrated spectrophotometric grade dimethylsulfoxide (Lot number FD021, Dojindo Laboratories) was added to each bacterial suspension at the ratio of 0.07 ml to 0.8 ml and stored at -80℃ until use. The genetic markers, such as auxotrophic mutation (histidine or tryptophan requirement), *rfa* mutation, *uvrA* or *uvrB* mutation, number of spontaneous revertants, R-factor, sensitivity of these bacterial strains to known mutagens were checked in advance before using the bacterial strains. And it was confirmed that the genetic markers and sensitivity of these bacterial strains were suitable.

1.4.2 Reason for the selection of the tester strains

The bacterial strains were chosen for the reason to take account of their high sensitivity to known mutagens, and these bacterial strains were the commonest tester strain to be used in this sort of the test.

1.5 Liver homogenate 9000×g fraction (S9 fraction)

The S9 fraction prepared from rat liver homogenates that had been treated with phenobarbital and 5,6-benzoflavone to induce drug-metabolizing enzymes was used. The S9 fraction was purchased from Kikkoman Corporation (Japan) and stored at -80℃ until use. The S9 fraction used in this test was less than 6 months after preparation. The appended data were as follows :

Species	Rat
Strain	Sprague-Dawley
Sex	Male
Age	7 weeks on arrival
Weight range	200～238 g
Lot number	RAA-518
Manufacture date	March 17, 2005

1.6 Media

The liquid medium of Nutrient broth No.2 (OXOID, Lot number 218041) was used for preparation of bacterial suspension. The minimum glucose agar plates used in this test were purchased from Kyokuto Pharmaceutical Industrial Co., Ltd. (Japan) and stored in the room temperature until use. The minimum glucose agar plates (Lot number DZA63401 prepared on March 4, 2005 and DZA64F01 prepared on April 15, 2005) were prepared in accordance with the guideline of the reverse mutation test using Microorganisms (JMHLW).

1.7 Overlay agar

The overlay agar used in the test of *Salmonella typhimurium* TA strains consisted of 10 part of the agar solution which include 0.6 wt% of agar (Bacto-Agar, Difco, Lot number 4076161) and 0.5 wt% of NaCl and of 1 part of the 0.5 mM D-biotin and L-histidine solution. In the case of the test using *Escherichia coli* WP2 *uvrA*, 0.5 mM D-biotin and L-histidine solution was replaced by 0.5 mM L-tryptophan solution.

2. Methods

2.1 Discrimination

The bacterial strain was distinguished by putting up the color seal or the indication of the color. The test number, the presence or the absence of S9Mix (metabolic activation) and the code number of the dose level were indicated on the minimum glucose agar plates.

2.2 Preparation of bacterial suspension

The frozen bacterial suspension was thawed, 100 µl of the thawed bacterial suspension was inoculated in 30 ml of Nutrient broth No.2 (OXOID) and stored in a refrigerator until the time of the cultivation start. The rest-thawed suspension used for inoculation was not reused. The bacterial suspension was then incubated at 37℃ for 10 hours with shaking. The optical density of this growing culture was measured with the photoelectric spectrophotometer (620 nm), and it was confirmed that it was approximately 1×10^9 cells per ml or more by converting the value of the optical density. The bacterial concentrations of the growing culture used in this test are shown in the following table :

($\times10^9$ cells per ml)

Strain	TA100	TA1535	WP2 *uvrA*	TA98	TA1537
Preliminary test	2.1	2.0	6.8	3.0	1.6
Main test	2.2	2.1	7.1	3.0	1.6
Confirmatory test	2.2	2.2	7.1	2.8	1.4

Preliminary test : preliminary reverse mutation test
Main test : main reverse mutation test
Confirmatory test : confirmatory reverse mutation test

2.3 Preparation of metabolic activation mixture (S9Mix)

The S9Mix was prepared by adding thawed S9 fraction to the cofactor solution at a concentration of 10 v/v% and then stored in a refrigerator until use. The composition of S9Mix per ml was as follows :

S9 fraction	10 v/v%
$MgCl_2$	8.0 µmol
KCl	33.0 µmol
G-6-P	5.0 µmol
NADPH	4.0 µmol
NADH	4.0 µmol
sodium phosphate buffer (pH7.4)	100.0 µmol

2.4 Preparation of positive controls

The positive control of NaN_3 was dissolved in distilled water (Lot number K4B78, Otsuka Pharmaceutical Factory, Inc.) and other positive controls were dissolved in dimethylsulfoxide (Lot number CEN4384, Wako Pure Chemical Industries, Ltd.). These positive control solutions were diluted in the used concentration and stored at -20℃ until use. The retention period of the positive control solution was less than 1 year, and the positive control solution that was diluted in the concentration to use was less than 3 months, respectively. It is confirmed that the positive controls stored are not declining the mutagenic activity for 1 year or more. The positive control used and the dose levels of each positive control are shown in the following table:

Strain	Absence of S9Mix (μg/plate)	Presence of S9Mix (μg/plate)
TA100	AF-2 (0.01)	B[*a*]P (5.0)
TA1535	NaN_3 (0.5)	2AA (2.0)
WP2 *uvrA*	AF-2 (0.01)	2AA (10.0)
TA98	AF-2 (0.1)	B[*a*]P (5.0)
TA1537	9AA (80.0)	B[*a*]P (5.0)

2.5 Selection of the solvent

Distilled water was used as the solvent of the test substance by the reason that the test substance was stable in water and that the solubility in water of the test substance was 10% or more.

2.6 Preparation of the test substance solution

The test substance of predetermined quantity was weighed when used. The highest concentration of the test substance solution used in this test was prepared using distilled water just before use. The solutions of the lower concentration were prepared by the same distilled water by serial dilution from the solution of the highest concentration. The weight value of the test substance was not converted into the purity in the time of the preparation.

2.7 Method

The reverse mutation test was composed of the main and the confirmatory test and the reappearance of the test results between the main and the confirmatory test was confirmed. The tests were performed in pre-incubation methods in all bacterial strains in both the presence and the absence of metabolic activation.

An amount of 0.1 ml of the test substance solution was put in the test tube. In the test of the absence of metabolic activation, 0.5 ml of 0.1 M phosphate buffer (pH7.4) and 0.1 ml of the bacterial suspension were added to the test substance solution and then incubated at 37℃ for 20 min with shaking. An amount of 0.5 ml of 0.1 M phosphate buffer was replaced by 0.5 ml of S9Mix in case of the presence of metabolic activation. The suspension was then thoroughly mixed with 2.0 ml of the overlay agar and the mixture was poured over the surface of the minimum glucose agar plate. The number of the minimum glucose agar plate of each concentration in the main and the confirmatory test was used in triplicate. Both the negative and the positive controls were similarly carried out. Sterility test of S9Mix, 0.1 M phosphate buffer, Nutrient broth No.2 and the test substance solution of the maximum concentration were performed to confirm the non-existence of the contaminant. After the incubation at 37℃ for 48 hours, the number of revertant colonies per plate was counted. The measuring of the number of the revertant colonies was carried out using colony analyzer that was set up to correct the measuring value by the correction method of miscount-correction. Toxic effect of the test substance was examined with a stereoscopic microscope and precipitation of the test substance was observed by naked eyes. The individual number of the revertant colonies, their mean value and standard deviation were expressed as the results of each dose level.

2.8 Dose levels

The preliminary test was performed to determine the most favorable dose levels of the test substance in the main and the confirmatory test. The number of the minimum glucose agar plate of the preliminary test was used in duplicate per each concentration and the test was performed in pre-incubation methods in all bacterial strains in both the presence and the absence of metabolic activation. The maximum dose of the preliminary test was set at 5000 μg/plate in accordance with the test guideline applied, and total six different dose levels with factor of 4 from the maximum dose were employed. The results of the preliminary test are shown in Tables 1 and 2.

The test substance produced toxic effect in all bacterial strains in both the presence and the absence of metabolic activation at the dose of 5000 μg/plate. However, two-fold or more increase in the number of the revertant colonies with dose-relativity was not observed in any bacterial strains at any dose levels regardless of the presence or the absence of metabolic activation. Furthermore, precipitation of the test substance was not detected in any dose levels regardless of the presence or the absence of metabolic activation. From the foregoing results, the maximum dose of the main and the confirmatory test in all bacterial strains in both the presence and the absence of metabolic activation was set at 5000 μg/plate and total six different dose levels with factor of 2 from the maximum dose were employed referring to the dose that toxic effect of the test substance was observed (Tables 3 to 6 and Tables 7 to 10, respectively).

2.9 Statistical analysis

Two statistical analyses of Dunnett's multiple comparison method (one-side test) and linear regression method were used in this test. The number of the revertant colonies of each bacterial strain of each dose was compared with the negative control in both of the presence and the absence of metabolic activation, and statistically significant difference in the number of the revertant colonies between those two groups was analyzed first by the multiple comparison method ($p<0.01$). When the statistical significant difference was obtained by the multiple comparison method, the specific dose-relativity was analyzed by linear regression method ($p<0.01$).

3. Evaluation of the test results

3.1 Acceptance criteria for the values of the negative control and the positive control

The number of the revertant colonies of the negative control and positive control in all bacterial strains was compared with the values of the historical data in our laboratory. The values of the negative control and the positive control were judged to satisfy acceptance criteria when either of the next two criteria were filled. One of the criteria is that the number of the revertant colonies is within the range of the standard value (mean ± 2SD) of the historical data. Another criterion is although the number of the revertant colonies is out of the range in the standard value, it is judged to be appropriate value because it occurs by chance from the comparison with the historical data.

3.2 Acceptance criteria for the test result

When the experiment satisfies the following criteria, it was judged that the test result was obtained from the test of the appropriate procedure.

1) The growth of the contaminant in the test materials, such as Nutrient broth, the test substance solution of the highest concentration, S9Mix, sodium phosphate buffer are not detected from the result of the sterility test.
2) The negative control values satisfy acceptance criteria.
3) The positive control values satisfy acceptance criteria. And the number of the revertant colonies of the positive control increases two-fold or more compared with the negative control.

3.3 Judgement of the test results

When the negative control and positive control values satisfy acceptance criteria and the test results satisfy acceptance criteria, the test substance was judged positive for mutagenic activity when biologically significant increases such as significant increases in the number of the revertant colonies with clear dose-relativity and reappearance was obtained.

4. Results and discussion

The results of the main and the confirmatory reverse mutation test are shown in Tables 3 to 6 and Tables 7 to 10, respectively. The dose-related curve indicated in Tables 3 to 6 is shown in Fig. 1 to 3.

The statistically significant difference from the negative control was not detected in any bacterial strains at any dose levels regardless of the presence or the absence of metabolic activation in the analysis of the reverse mutation test with the Dunnett's multiple comparison method. And the reappearance of the test results between the main and the confirmatory test was confirmed. From these results, it was judged that the test substance was not inducible for mutagenicity. On the other hand, the growth of the contaminant was not observed in a result of the sterility test. The numbers of the revertant colonies of the negative control and the positive control were within the range of the standard value of the historical data in our laboratory. Furthermore, all of 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide, sodium azide, 9-aminoacridine, benzo[*a*]pyrene and 2-aminoanthracene used as the positive controls increased the number of the revertant colonies two-fold or more compared with the negative control with all bacterial strains, respectively. These results indicate that the test has been properly carried out. The environmental factor that might influence the reliability of this test and deviation from the protocol were not detected.

It is concluded from the foregoing results that the mutagenic activity of the test substance is considered negative under the test conditions employed.

The test substance produced toxic effect in all bacterial strains in both the presence and the absence of metabolic activation at the dose of 5000 μg/plate. The precipitation of the test substance was not detected at any dose levels regardless of the presence or the absence of metabolic activation.

5. Storage of the records

All of the records that related to this test will be stored at archives of

records are stored for 10 years after the completion of the test, after the notification to JMHLW is accepted, or after the notice to apply to the regulation of JMHLW, JMETI and JMOE is done. The contents of the records are as follows. Storage of the records will be decided by discussion between the sponsor and Genetic Laboratory, JBS, Inc.

Storage of the records

Protocol
Raw data
Records on the test substance and control substance
Test substance
Records of Quality Assurance Unit
Final report
Other documents specified by the GLP standards

6. Good Laboratory Practice (GLP) and the test guidelines that were applied to the test

< Good Laboratory Practice >

1. The standard that the Minister of Labor establishes based on the Industrial Safety and Health Law Article 34-3 Paragraph 2 (Notification No. 76 of JMOL on September 1, 1988) and Notification on partial revision of the standard (Notification No. 13 of JMOL on March 29, 2000).
2. Notification for concerning testing facilities conducting the test related to the new chemical substances (Notification No. 1121003 of JMHLW, No. 3 of JMETI, No. 031121004 of JMOE on November 21, 2003).

< Test guidelines >

1. The standard that the Minister of Labor establishes based on the Industrial Safety and Health Law Article 57-2 Paragraph 1 (Notification No. 77 of JMOL on September 1, 1988) and Notification on partial revision of the standard (Notification No. 67 of JMOL on June 2, 1997).
2. Notification of testing methods relating to the new chemical substances (Notification No. 1121002 of JMHLW, No. 2 of JMETI, No. 031121002 of JMOE on November 21, 2003).
3. OECD Guideline for Testing of Chemicals 471, "Bacterial Reverse Mutation Test" (21 July, 1997).

Table 1 Preliminary reverse mutation test of
Salmonella typhimurium and *Escherichia coli* in the absence of metabolic activation

Exp.No. 12923

Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean)											
		Base-pair substitution type						Frameshift type					
		TA100		TA1535		WP2 *uvrA*		TA98		TA1537			
D.W.		127		12		25		22		6			
		124	(126)	8	(10)	24	(25)	19	(21)	7	(7)		
	5	127		7		22		18		4			
		130	(129)	7	(7)	22	(22)	24	(21)	5	(5)		
	20	119		8		24		17		8			
		125	(122)	9	(9)	20	(22)	21	(19)	9	(9)		
	78	130		11		24		18		4			
		119	(125)	9	(10)	26	(25)	20	(19)	4	(4)		
	313	107		11		23		25		4			
		123	(115)	6	(9)	25	(24)	18	(22)	7	(6)		
	1250	127		6		26		20		4			
		134	(131)	12	(9)	21	(24)	21	(21)	8	(6)		
	5000	81 *		2 *		15 *		15 *		1 *			
		81 *	(81)	4 *	(3)	15 *	(15)	13 *	(14)	5 *	(3)		
AF-2	0.01	633				134							
		659	(646)			113	(124)						
	0.1							545					
								514	(530)				
NaN_3	0.5			439									
				362	(401)								
9AA	80.0									564			
										537	(551)		

D.W. : distilled water
AF-2 : 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
NaN_3 : sodium azide
9AA : 9-aminoacridine

* : Toxic effect of the test substance was observed.

Table 2 Preliminary reverse mutation test of
Salmonella typhimurium and *Escherichia coli* in the presence of metabolic activation

Exp.No. 12923

Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean)									
		Base-pair substitution type						Frameshift type			
		TA100		TA1535		WP2 *uvrA*		TA98		TA1537	
D.W.		123 136	(130)	12 7	(10)	25 26	(26)	25 20	(23)	8 11	(10)
	5	120 121	(121)	9 6	(8)	25 21	(23)	23 23	(23)	11 8	(10)
	20	131 120	(126)	5 8	(7)	29 20	(25)	21 25	(23)	5 6	(6)
	78	137 144	(141)	9 7	(8)	22 34	(28)	29 23	(26)	8 5	(7)
	313	137 117	(127)	12 8	(10)	25 24	(25)	24 21	(23)	7 8	(8)
	1250	133 133	(133)	12 7	(10)	27 26	(27)	30 20	(25)	7 9	(8)
	5000	61 * 82 *	(72)	1 * 3 *	(2)	11 * 13 *	(12)	16 * 11 *	(14)	3 * 5 *	(4)
B[*a*]P	5.0	1032 1046	(1039)					309 270	(290)	89 86	(88)
2AA	2.0			215 227	(221)						
	10.0					782 846	(814)				

D.W. : distilled water
B[*a*]P : benzo[*a*]pyrene
2AA : 2-aminoanthracene

* : Toxic effect of the test substance was observed.

Table 3 Main reverse mutation test of .
pair substitution detectable strains of *Salmonella typhimurium* TA100, TA1535 and *Escherichia coli* WP2 *uvrA* in the absence of metabolic activation

Exp. No. 12923

Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean ± standard deviation)					
		TA100		TA1535		WP2 *uvrA*	
D.W.		124 133 128	(128 ± 4.5)	11 12 8	(10 ± 2.1)	30 30 27	(29 ± 1.7)
	156	132 126 130	(129 ± 3.1)	8 9 9	(9 ± 0.6)	32 32 29	(31 ± 1.7)
	313	102 108 120	(110 ± 9.2)	10 9 11	(10 ± 1.0)	30 33 35	(33 ± 2.5)
	625	131 135 106	(124 ± 15.7)	7 11 8	(9 ± 2.1)	29 32 34	(32 ± 2.5)
	1250	125 122 124	(124 ± 1.5)	9 7 7	(8 ± 1.2)	33 30 29	(31 ± 2.1)
	2500	131 122 117	(123 ± 7.1)	8 7 9	(8 ± 1.0)	26 28 25	(26 ± 1.5)
	5000	84 * 57 * 66 *	(69 ± 13.7)	2 * 1 * 1 *	(1 ± 0.6)	14 * 12 * 17 *	(14 ± 2.5)
AF-2	0.01	457 477 492	(475 ± 17.6)			106 113 114	(111 ± 4.4)
NaN_3	0.5			453 424 463	(447 ± 20.3)		

D.W. : distilled water
AF-2 : 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
NaN_3 : sodium azide

* : Toxic effect of the test substance was observed.

† : Significant difference from the negative control ($p<0.01$)

Table 4 Main reverse mutation test of .
using frameshift mutation detectable strains of *Salmonella typhimurium* TA98 and TA1537 in the absence of metabolic activation

Exp. No. 12923

Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean ± standard deviation)			
		TA98		TA1537	
D.W.		25 27 25	(26 ± 1.2)	6 7 6	(6 ± 0.6)
	156	22 26 28	(25 ± 3.1)	5 5 8	(6 ± 1.7)
	313	27 29 25	(27 ± 2.0)	8 7 6	(7 ± 1.0)
	625	23 28 30	(27 ± 3.6)	5 6 8	(6 ± 1.5)
	1250	26 29 32	(29 ± 3.0)	9 6 7	(7 ± 1.5)
	2500	26 28 29	(28 ± 1.5)	7 5 6	(6 ± 1.0)
	5000	8 * 5 * 8 *	(7 ± 1.7)	3 * 1 * 2 *	(2 ± 1.0)
AF-2	0.1	498 460 502	(487 ± 23.2)		
9AA	80.0			517 404 414	(445 ± 62.6)

D.W. : distilled water
AF-2 : 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
9AA : 9-aminoacridine

* : Toxic effect of the test substance was observed.

† : Significant difference from the negative control ($p<0.01$)

Table 5 Main reverse mutation test of using base-pair substitution detectable strains of *Salmonella typhimurium* TA100, TA1535 and *Escherichia coli* WP2 *uvrA* in the presence of metabolic activation

Exp. No. 12923

Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean ± standard deviation)		
		TA100	TA1535	WP2 *uvrA*
D.W.		142 138 139 (140 ± 2.1)	10 10 9 (10 ± 0.6)	32 33 26 (30 ± 3.8)
	156	133 121 129 (128 ± 6.1)	9 12 7 (9 ± 2.5)	29 33 35 (32 ± 3.1)
	313	142 145 133 (140 ± 6.2)	8 9 9 (9 ± 0.6)	28 27 35 (30 ± 4.4)
	625	122 140 148 (137 ± 13.3)	8 8 7 (8 ± 0.6)	30 28 36 (31 ± 4.2)
	1250	139 129 132 (133 ± 5.1)	9 10 8 (9 ± 1.0)	34 36 36 (35 ± 1.2)
	2500	129 135 129 (131 ± 3.5)	7 8 6 (7 ± 1.0)	28 32 33 (31 ± 2.6)
	5000	71 * 73 * 87 * (77 ± 8.7)	1 * 2 * 2 * (2 ± 0.6)	20 * 15 * 11 * (15 ± 4.5)
B[*a*]P	5.0	1019 1099 1065 (1061 ± 40.1)		
2AA	2.0		203 234 226 (221 ± 16.1)	
	10.0			848 806 797 (817 ± 27.2)

D.W. : distilled water
B[*a*]P : benzo[*a*]pyrene
2AA : 2-aminoanthracene

* : Toxic effect of the test substance was observed.

† : Significant difference from the negative control ($p<0.01$)

Table 6 Main reverse mutation test of frameshift mutation detectable strains of *Salmonella typhimurium* TA98 and TA1537 in the presence of metabolic activation

Exp. No. 12923

Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean ± standard deviation)			
		TA98		TA1537	
D.W.		28 26 30	(28 ± 2.0)	7 5 8	(7 ± 1.5)
	156	33 26 23	(27 ± 5.1)	8 6 7	(7 ± 1.0)
	313	28 32 29	(30 ± 2.1)	5 6 8	(6 ± 1.5)
	625	30 29 33	(31 ± 2.1)	7 5 8	(7 ± 1.5)
	1250	34 32 29	(32 ± 2.5)	6 6 8	(7 ± 1.2)
	2500	25 23 26	(25 ± 1.5)	9 7 7	(8 ± 1.2)
	5000	21 * 19 * 13 *	(18 ± 4.2)	1 * 4 * 2 *	(2 ± 1.5)
B[*a*]P	5.0	265 325 340	(310 ± 39.7)	82 79 76	(79 ± 3.0)

D.W. : distilled water
B[*a*]P : benzo[*a*]pyrene

* : Toxic effect of the test substance was observed.

† : Significant difference from the negative control ($p<0.01$)

Table 7 Confirmatory reverse mutation test of base-pair substitution detectable strains of *Salmonella typhimurium* TA100, TA1535 and *Escherichia coli* WP2 *uvrA* in the absence of metabolic activation

Exp. No. 12923

Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean ± standard deviation)		
		TA100	TA1535	WP2 *uvrA*
D.W.		120 119 108 (116 ± 6.7)	9 8 9 (9 ± 0.6)	26 28 24 (26 ± 2.0)
	156	124 126 132 (127 ± 4.2)	8 10 7 (8 ± 1.5)	30 28 33 (30 ± 2.5)
	313	135 107 128 (123 ± 14.6)	11 10 9 (10 ± 1.0)	23 29 23 (25 ± 3.5)
	625	133 120 128 (127 ± 6.6)	8 8 9 (8 ± 0.6)	34 25 28 (29 ± 4.6)
	1250	118 132 129 (126 ± 7.4)	7 9 9 (8 ± 1.2)	22 26 26 (25 ± 2.3)
	2500	109 128 106 (114 ± 11.9)	8 7 7 (7 ± 0.6)	25 27 23 (25 ± 2.0)
	5000	99 * 77 * 65 * (80 ± 17.2)	3 * 2 * 3 * (3 ± 0.6)	15 * 10 * 14 * (13 ± 2.6)
AF-2	0.01	480 518 510 (503 ± 20.0)		114 135 106 (118 ± 15.0)
NaN_3	0.5		508 456 435 (466 ± 37.6)	

D.W. : distilled water
AF-2 : 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
NaN_3 : sodium azide

* : Toxic effect of the test substance was observed.

† : Significant difference from the negative control ($p<0.01$)

Table 8 Confirmatory reverse mutation test of using frameshift mutation detectable strains of *Salmonella typhimurum* TA98 and TA1537 in the absence of metabolic activation

Exp. No. 12923

Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean ± standard deviation)			
		TA98		TA1537	
D.W.		22 25 26	(24 ± 2.1)	7 6 8	(7 ± 1.0)
	156	23 21 25	(23 ± 2.0)	8 7 9	(8 ± 1.0)
	313	26 22 26	(25 ± 2.3)	7 9 9	(8 ± 1.2)
	625	23 26 24	(24 ± 1.5)	8 7 7	(7 ± 0.6)
	1250	25 26 28	(26 ± 1.5)	8 6 9	(8 ± 1.5)
	2500	21 24 24	(23 ± 1.7)	7 6 8	(7 ± 1.0)
	5000	8 * 9 * 4 *	(7 ± 2.6)	1 * 1 * 1 *	(1 ± 0.0)
AF-2	0.1	590 517 547	(551 ± 36.7)		
9AA	80.0			424 415 338	(392 ± 47.3)

D.W. : distilled water
AF-2 : 2-(2-furyl)-3-(5-nitro-2-furyl)acrylamide
9AA : 9-aminoacridine

* : Toxic effect of the test substance was observed.

† : Significant difference from the negative control ($p<0.01$)

Table 9 Confirmatory reverse mutation test of base-pair substitution detectable strains of *Salmonella typhimurium* TA100, TA1535 and *Escherichia coli* WP2 *uvrA* in the presence of metabolic activation

Exp. No. 12923

Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean ± standard deviation)					
		TA100		TA1535		WP2 *uvrA*	
D.W.		128 123 125	(125 ± 2.5)	8 9 9	(9 ± 0.6)	34 25 28	(29 ± 4.6)
	156	128 118 134	(127 ± 8.1)	9 10 8	(9 ± 1.0)	36 26 27	(30 ± 5.5)
	313	133 123 137	(131 ± 7.2)	8 8 9	(8 ± 0.6)	35 33 25	(31 ± 5.3)
	625	125 134 133	(131 ± 4.9)	12 8 8	(9 ± 2.3)	30 27 29	(29 ± 1.5)
	1250	123 122 137	(127 ± 8.4)	7 8 7	(7 ± 0.6)	26 33 29	(29 ± 3.5)
	2500	105 107 139	(117 ± 19.1)	8 6 9	(8 ± 1.5)	33 33 26	(31 ± 4.0)
	5000	72 * 53 * 89 *	(71 ± 18.0)	2 * 2 * 1 *	(2 ± 0.6)	25 * 22 * 17 *	(21 ± 4.0)
B[*a*]P	5.0	1044 1069 1104	(1072 ± 30.1)				
2AA	2.0			204 206 199	(203 ± 3.6)		
	10.0					752 717 897	(789 ± 95.4)

D.W. : distilled water
B[*a*]P : benzo[*a*]pyrene
2AA : 2-aminoanthracene

* : Toxic effect of the test substance was observed.

† : Significant difference from the negative control ($p<0.01$)

Table 10 Confirmatory reverse mutation test of using frameshift mutation detectable strains of *Salmonella typhimurium* TA98 and TA1537 in the presence of metabolic activation

Exp. No. 12923

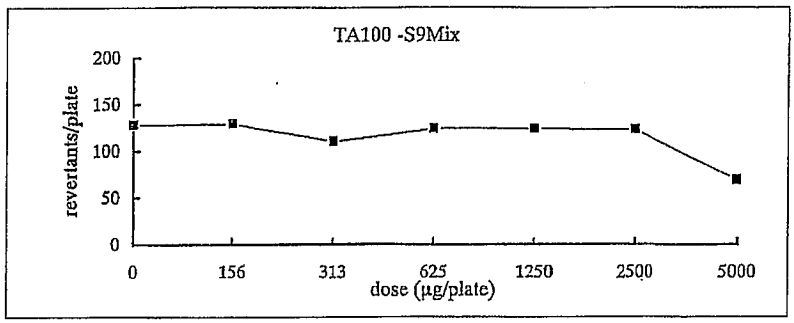
Compound	Dose (μg/plate)	Number of revertant colonies per plate (mean ± standard deviation)			
		TA98		TA1537	
D.W.		27 25 27	(26 ± 1.2)	9 11 7	(9 ± 2.0)
	156	26 22 23	(24 ± 2.1)	8 7 6	(7 ± 1.0)
	313	28 25 26	(26 ± 1.5)	10 8 7	(8 ± 1.5)
	625	21 23 26	(23 ± 2.5)	9 6 9	(8 ± 1.7)
	1250	25 26 24	(25 ± 1.0)	8 9 7	(8 ± 1.0)
	2500	23 29 27	(26 ± 3.1)	7 5 7	(6 ± 1.2)
	5000	20 * 13 * 14 *	(16 ± 3.8)	3 * 1 * 2 *	(2 ± 1.0)
B[*a*]P	5.0	295 324 384	(334 ± 45.4)	85 78 81	(81 ± 3.5)

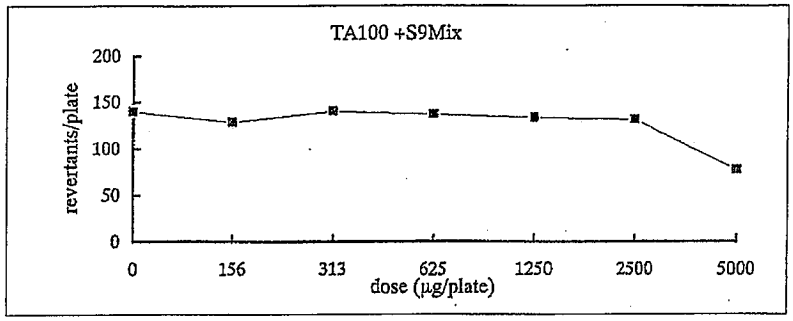
D.W. : distilled water
B[*a*]P : benzo[*a*]pyrene

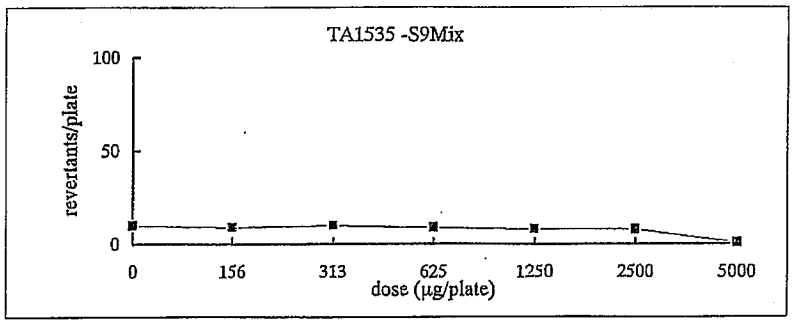
* : Toxic effect of the test substance was observed.

† : Significant difference from the negative control ($p<0.01$)

Fig. 1 Dose-response curve (Exp. No. 12923)







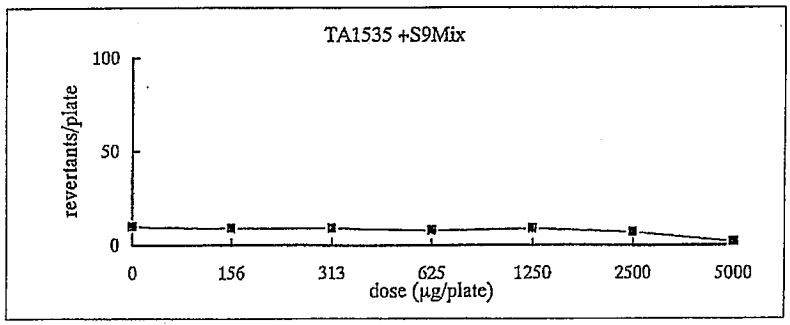
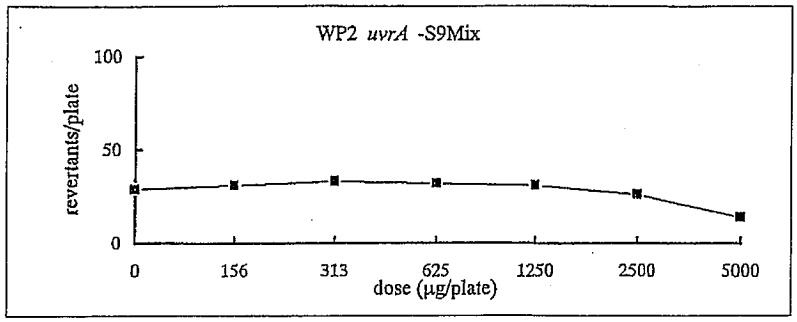
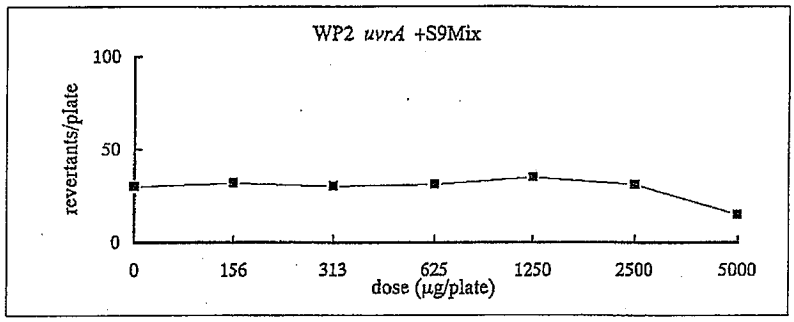
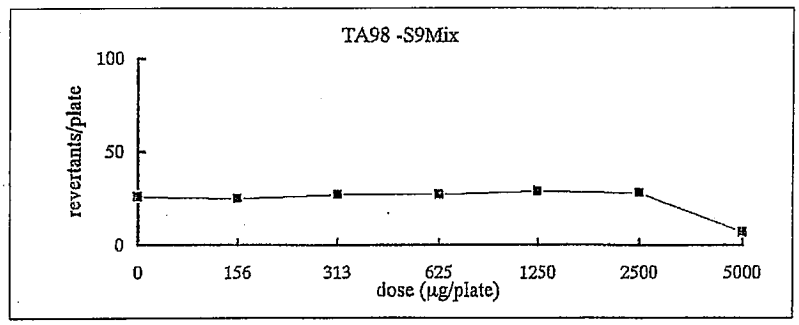


Fig. 2 Dose-response curve (Exp. No. 12923)







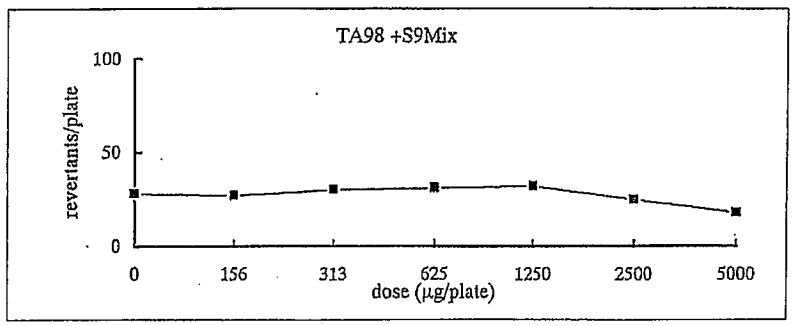
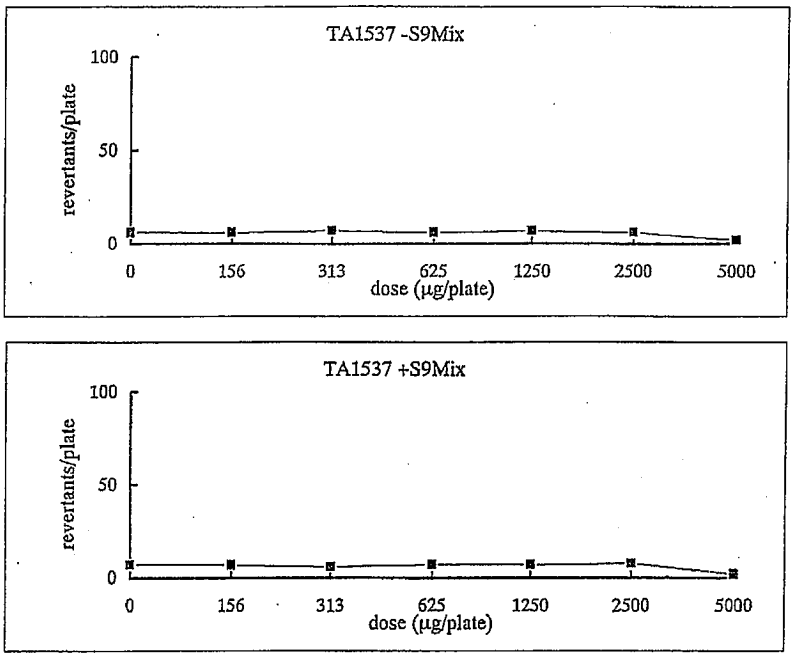


Fig. 3 Dose-response curve (Exp. No. 12923)



HISTORICAL DATA

Pre-incubation method

Control substance	Strain	S9Mix	Historical data						
			Number	Mean	S.D.	Min.	Max.	Standard value	
negative controls	TA100	-	798	111	11	79	165	90	~ 133
		+	770	122	13	89	177	96	~ 147
	TA1535	-	764	9	2.7	2	22	3	~ 14
		+	740	9	2.6	2	22	4	~ 14
	WP2 *uvrA*	-	730	23	4.8	10	47	14	~ 33
		+	710	25	5.0	9	44	15	~ 35
	TA98	-	835	23	4.0	11	36	15	~ 31
		+	805	29	5.5	11	51	18	~ 40
	TA1537	-	754	8	2.3	1	19	3	~ 12
		+	730	9	3.1	1	25	2	~ 15
positive controls	TA100	-	750	544	72.8	399	853	398	~ 689
		+	720	1150	153.5	753	1960	843	~ 1457
	TA1535	-	698	536	92.0	223	848	352	~ 720
		+	670	218	51.0	125	431	116	~ 320
	WP2 *uvrA*	-	641	107	14.8	51	193	78	~ 137
		+	620	812	144.4	351	1253	523	~ 1101
	TA98	-	757	561	58.8	354	796	444	~ 679
		+	727	349	49.8	82	559	249	~ 448
	TA1537	-	680	574	160.4	136	1229	254	~ 895
		+	656	78	13.1	43	138	52	~ 104

Number : total number of counted data
S.D. : standard deviation
Standard value : mean$\pm$2$\times$S.D.

Positive control substance :
S. typhimurium TA98 ; AF2 (-S9Mix), B[*a*]P (+S9Mix)
S. typhimurium TA100 ; AF2 (-S9Mix), B[*a*]P (+S9Mix)
S. typhimurium TA1535 ; NaN_3 (-S9Mix), 2AA (+S9Mix)
S. typhimurium TA1537 ; 9AA (-S9Mix), B[*a*]P (+S9Mix)
E. coli WP2 *uvrA* ; AF2 (-S9Mix), 2AA (+S9Mix)

AUTHENTIFICATION

Title : Reverse mutation test of microorganisms.

Test number : 12923

I prove that this report was translated precisely from the final report that was written in Japanese.